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(19) (CA) **CANADIAN PATENT** (12)

(54) Semen Sexing

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U.K.

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ABSTRACT:

In a method for sorting spermatozoa, spermatozoa are stained with Hoechst 33342. The fluorescence distribution of stained spermatozoa is complex: non-motile spermatozoa display a higher fluorescence than motile spermatozoa. The fluorescence profile of the motile spermatozoa is bimodal, and enables the spermatozoa to be sorted into distinct populations of motile spermatozoa.

SEMEN SEXING

The present invention relates to a method of sorting living spermatozoa, and, for example, to a method of sorting living spermatozoa according to sex; that is, according to whether the spermatozoa bear an X or Y chromosome.

Throughout the following description, the lower case letters in parentheses refer to the following:

(a) Almquist, J.O., Flipse, R.J. & Thacker, D.L. (1954) Diluters of bovine semen, IV. Fertility of bovine spermatozoa in heated homogenized milk and skimmed milk. J. Dairy Sci. 37 1303-1304.

(b) Dean, P.N., Pinkel, D. & Mendelson, M.L. (1978) Hydrodynamic orientation of sperm heads for flow cytometry. Biophys. J.23, 7-13.

(c) First, N.L. (1971) Collection and preservation of sperm. In "Methods in Mammalian Embryology", p. 15-35. Ed. J.C. Daniel, Jr. Freeman, San Francisco.

(d) Fulwyler, M.J. (1977) Hydrodynamic orientation of cells. J. Histochem. Cytochem. 25, 781-783.

(e) Gledhill, B.L., Lake, S. & Dean, P.N. (1979) Flow cytometry and sorting of sperm and other male germ cells. In Flow Cytometry and Sorting, pp.

471-485. Eds M.R. Melamed. P.F. Mullaney & M.L. Mendelsohn. Wiley, New York.

(f) Herzenberg, L.A. & Herzenberg, L.A. (1978) Analysis and separation using the fluorescence activated cell sorter. In "Handbook of Experimental Immunology," 3rd edn, pp. 22.1 - 22.21. Ed. D.M. Weir. Blackwell Scientific Publications, Oxford.

(g) Herzenberg, L.A., Sweet, R.G., & Herzenberg, L.A. (1976) Fluorescence activated cell sorting. Sci. Am. 234, 108-117.

(h) Klasen, M. & Schmidt, M. (1981) An improved method for Y body identification and confirmation of a high incidence of YY sperm nuclei. Hum. Genet. 58, 156-161.

(i) Loken, M.R., Parks, D.R. & Herzenberg, L.A. (1977) Identification of cell asymmetry and orientation by light scattering. J. Histochem. Cytochem. 25, 790-795.

(j) Lydon, M.J., Keeler, K.D. & Thomas, D.B. (1980) Vital DNA staining and cell sorting by flow micro-fluorometry. J. cell. Physiol. 102. 175-181.

(k) Muller, W. & Gautier, F. (1975) Interaction of heteroaromatic compounds with nucleic acid A-T specific non-intercalating DNA ligands. Eur. J. Biochem. 54, 385-394.

(l) Russell, W.C., Newman, C. & Williamson, D.H. (1975) A simple cytochemical technique for demonstration of DNA in cells infected with mycoplasma and viruses. Nature, Lond. 253, 461-462.

(m) Stovel, R.T., Sweet, R.G. & Herzenberg, L.A. (1978) A means for orienting flat cells in flow systems. Biophys. J. 23, 1-5.

(n) Szabo, G., Jr, Kiss, A. & Damjanovich, S. (1981) Flow cytometric analysis of the uptake of Hoechst 33342 dye by human lymphocytes. Cytometry 2. 20-23.

(o) Tobey, R.A. & Crissman, H.A. (1975) Unique techniques for microfluorometry. Expl. Cell Res. 93, 235-239.

(p) Van Dilla, M.A., Gledhill, B.L, Lake, S., Dean, P.N., Gray, J.W., Kachel, V., Barlogie, B. & Gohde, W. (1977) Measurement of mammalian sperm deoxyribonucleic acid by flow cytometry. Problems and approaches. J. Histochem. Cytochem. 25, 763-773.

Flow microfluorometry is a convenient method for measuring the DNA content of mammalian cells (o). Spermatozoa, by virtue of their ease of collection from many species, their homogeneity and their haploidy, are particularly suitable for such studies (p;e). To date, the majority of studies of the DNA content of spermatozoa have been carried out using fixed material stained with fluorochromes such as acridine orange, ethidium bromide, or mithramycin. Recently, the bisbenzimidazole dyes Hoechst® 33258, Hoechst® 33342, and DAPI(4',6'-diamidino-2-phenylindole) have been introduced as quantitative fluorescent stains for DNA. These dyes, although they bind tightly to DNA, do not intercalate into the molecule and hence are reputed not to disrupt its structure (k;l). These fluorochrome dyes are consequently capable of being used as quantitative vital stains

for DNA: Hoechst 33258 and Hoechst 33342 have been used as vital stains to distinguish phases of the cell cycle.

Since spermatozoa are tail bearing and motile they orientate with their long axis along the line of flow in a flow microfluorometry system (p). It has been concluded that an apparent bimodal DNA distribution in fixed acriflavine/Feulgen-stained bull sperm heads analyzed in such a system, is due to an orientation artefact (b), perhaps analogous to that previously described in (i) for the light scatter (size) artefact seen with chicken red blood cells (chicken RBC). Both of these artefacts can be by-passed or removed by the use of an appropriate nozzle which will control the orientation of flattened particles such as sperm heads or chicken RBC relative to the laser beam of the flow microfluorometry system (m ; b). As an alternative approach, distribution artefacts can be tested by sorting the population into its separate components and then reanalyzing them independently: if an artefact is involved, each reanalyzed peak will give a bimodal peak similar to that observed originally.

Various aspects of the invention are as follows:

A method of sorting living spermatozoa, the method comprising: the vital staining of spermatozoa, with a fluorochrome dye; subjecting the stained spermatozoa to a light source which causes fluorescence; and sorting the spermatozoa according to the fluorescence intensities associated therewith.

A method of sorting living spermatozoa, the method comprising: the vital staining of spermatozoa with a fluorochrome dye which binds tightly to DNA, does not intercalate the DNA molecule of chromosomes and hence does not disrupt the structure of DNA molecules; subjecting the spermatozoa to a light source which causes fluorescence and sorting the spermatozoa into different groups according to the fluorescence intensities associated therewith, one group mainly comprising X-chromosome bearing spermatozoa; and another group mainly comprising Y-chromosome bearing spermatozoa.

The dye may be a bisbenzimidazole dye.

In an embodiment of the invention, the bisbenzimidazole dye Hoechst 33342 is used as a vital fluorescent stain for DNA which allows spermatozoa to remain motile after analysis. The fluorescence may be examined in detail using a commercially

available fluorescence-activated cell sorter.

For a better understanding of the present invention, and to show how the same may be carried into effect, reference will now be made, by way of example, to the accompanying drawings in which:

FIGURE 1 is a graph showing the distribution of fluorescence of bull spermatozoa stained with Hoechst 33342;

FIGURE 2 is a graph showing the distribution of Figure 1, with a higher gain setting for the fluorescence-activated cell sorter;

FIGURE 3 is a graph showing the distribution of cockerel spermatozoa stained with Hoechst 33342 (5 µg/ml) in egg medium;

FIGURE 4 is a graph showing reanalysis of the peaks AI and AII in Figure 2;

FIGURES 5a to 5c are graphs showing the results of analysis with different orientations of the cells; and

FIGURE 6 is a table showing the effect of an orientating nozzle on FACS analysis of chicken RBC (size) and bull spermatozoa (fluorescence) compared to non-orientated cells.

In preparation for the analysis semen is collected, using an appropriate artificial vagina (c), from Fresian and Hereford bulls. Shortly after ejaculation, semen is added to 1-2 volumes of egg or milk medium at 20-22°C. Milk medium is made according to the method described in (a), which comprises: centrifuging pasteurized milk at 2000 g for 10 min; removing the cream; taking the underlying fat-free liquid from this slow speed spin; and pelleting the milk solids by centrifugation at 48000 g for 30 mins. The clear supernatant is then heated at 92-96°C for 10 min, and 0.125 g D-fructose/ml and antibiotics (10^4 units penicillin + 10 mg streptomycin sulphate per 100 ml) is added when the supernatant has cooled.

The spermatozoa are washed twice by centrifugation

at 1000 g for 5 min followed by gentle resuspension of the pellet in sufficient fresh medium to give a concentration of, for example, 5 x 10^6 spermatozoa/ml.

Intact spermatozoa are then stained with Hoechst 33342 in milk medium at a concentration of 2 µg/ml for bull spermatozoa and 5 µg/ml for cockerel spermatozoa, at room temperature for 2-3 hours. The dye concentrations may be determined empirically from subjective assessment of optimal staining without overt cytotoxicity.

Flow microfluorometric analysis (g) is carried out using a fluorescence Activated Cell Sorter (such as, for example, FACS II:Becton Dickinson Electronics Laboratories, Sunnyvale, California). The light source for the FACS may be a 164-05 ultra violet-enhanced argon-ion laser, (Spectraphysics), operated at 20 mW in the u.v. range of wavelengths. Right-angle scatter of u.v. laser light is prevented from entering the fluorescence detector by a Wratten 2B filter. The FACS is calibrated in the u.v. using glutaraldehyde-fixed chicken red blood cells (f).

Samples of spermatozoa are analysed and sorted at room temperature (20-22°C) at a rate of up to 3500-5000 cells/sec, except during orientation experiments in which the rate was reduced to <800 cells/sec. The sheath fluid is Dulbecco's phosphate-buffered saline (pH 7.2; containing Mg^{2+} and Ca^{2+}), but without stain.

The total fluorescence is calculated (in arbitrary units), for example by a computer. Such a computer is an LSI-11 based mini computer (Digital Equipment Corporation, MA, USA) linked to the FACS, which calculates the total fluorescence between channels 1 and 256 as follows (I):

$$\text{Total fluorescence} = \sum_{i}^{256} \frac{\text{no. of cells in a channel} \times \text{channel no.}}{100} \qquad \text{(I)}$$

Cells can be orientated in a single vertical plane

at a predetermined angle to the laser beam by the method described in (m). A (wedge shaped) sample injection tube, with faces set at 20°C to the axis flow, has the effect of making a (central) stream ribbon-shaped within the sheath stream. Since the velocity of the sheath stream is considerably higher than that of the sample stream, the latter is drawn into a thin ribbon and the flattened cells within this sample become orientated into the plane of the ribbon.

Extrapolating from maximal flow rates which allow successful orientation of chicken red blood cells, it has been estimated, on the basis of cell (head) size and viscosity of the medium, that successful orientation of spermatozoa should occur providing that the flow rate does not exceed 800 cells/sec, when using a sample density of 5×10^6/ml.

When necessary, heads may be removed from the spermatozoa in milk medium by ultra-sonication for 5-10 min in a MSE ultrasonicator.

A population of bull spermatozoa stained for a minimum of 2 hours with Hoechst 33342, (2 µg/ml Hoechst 33342) in milk medium shows a complex distribution of fluorescence intensity, which is illustrated in Figure 1. Data are given for spermatozoa in milk medium at ambient temperature (20-23°C) for 2 hours and those killed by being heated to 56°C for 5 min. There are two pairs of peaks in the distribution, which have been labelled A and B respectively. When examined microscopically, cells from window B are non- (or only partly) motile, whereas spermatozoa sorted from window A show active forward motility. The likelihood that the B peaks represent dead or moribund spermatozoa was tested by submitting a sample of stained spermatozoa to 56°C for 5 min. This treatment left the spermatozoa totally immotile and when the fluorescence distribution of these immotile spermatozoa was examined the entire distribution was concentrated in the B peaks. A small peak seen between A and B in Figure

1 may represent spermatozoa in a transitory state between A and B or the presence of a small percentage of diploid spermatozoa (h).

Attention was concentrated on the A peaks of the fluorescence distribution of stained bull spermatozoa by running the FACS fluorescence gain at a higher setting (Figure 2) so that the B peaks moved off-scale. The low and high peaks of the observed bimodal fluorescence distribution of the A peaks (AI and AIII) contained approximately equal numbers of spermatozoa. The average fluorescence of spermatozoa in peak AII was approxiamtely 30% higher than that in peak AI.

Qualitatively similar bimodal distributions are also obtained using the same procedures as outlined above for the bull, when analysing ejaculated rabbit, sheep, goat and human spermatozoa.

When cockerel spermatozoa (~0.5 x 4 µm heads, ~ 8 µm tails) were stained with H33342 the resulting fluorescence profile was quite different from that of bull spermatozoa (Figure 3). The monophasic distribution of fluorescence may reflect either the homogametic nature of male birds or be due to the absence of an orientation artefact in the cylindrically headed cockerel spermatozoa. The bimodal fluorescence distribution of bull spermatozoa may be due to a machine artefact, analogous to that observed for light scatter (size) analysis of chicken red blood cells, but reflect underlying biological or physiological differences. An investigation into the nature of the observed bimodality was carried out by an analysis-sort-reanalysis of stained spermatozoa and by the use of an "orientating" nozzle.

First, the living, Hoechst 33342-stained bull spermatozoa with a fluorescence distribution similar to that shown in Figure 2, were physically separated (sorted) into AI and AII population. Each separated population was then re-analysed and the respective fluorescence distributions are shown in Figure 4.

Although the peaks were not clearly unimodal, the spermatozoa from the AII fraction had a higher overall fluorescence than those from AI as would be expected if the spermatozoa in peak AI were from a population different from that of those in peak AII. The low fluorescent peak appearing at approximately channel 30 for both populations in Figure 4 was due to spermatozoa from which the H33342 had leached. Fixation of spermatozoa with buffered formal-saline (pH 7.4) before or after staining or after they had been sorted failed to reduce the leakage of dye. In 17 experiments in which the spermatozoa in peaks AI and AII were separated, the total fluorescence intensity of the reanalysed AII population was 15.6 $\pm$ 2.9% greater than that of the AI population. For a comparison, the same experiment was performed using chicken RBC. It is known that the apparent bimodal size distribution of the chicken RBC is an artefact related to the orientation of individual cells to the laser beam. When the chicken RBC were sorted into two peaks on the basis of scatter, each separated peak gave the same bimodal distribution as the original, unsorted, material when reanalysed.

Second, an orientation nozzle similar to that decribed in (m) was used to analyse bull spermatozoa. The efficiency of the nozzle was tested using a light-scatter analysis of chicken RBC (1200 cells/sec). Figure 5 shows results using an orientating nozzle for (a) chicken RBC and (b, c) bull spermatozoa. In Figure 5a) peak 1 was obtained when the sample ribbon was parallel to the laser beam; peak 2 was obtained when the sample ribbon was at right angles to the laser beam; and peak 3 for randomly orientated cells. In Figure 5b) peak 1 was obtained when the heads of the spermatozoa were orientated edge on with respect to the laser beam and peak 2 when the sample was rotated through 90° in the axis of the flow (laser beam intersecting the broad side of head); randomly orientated cells are indicated by 3.

In Figure 5c) the bimodal distribution of fluorescence intensity of intact Hoechst 33342-stained bull spermatozoa was not affected by altering the orientation of the sample ribbon: the distributions of randomly orientated cells overlapped. The scatter distribution of chicken RBC (Figure 5a) was affected by orientating the cells with their edges parallel to or at right angles to the laser beam. A similar effect was observed when sperm heads were passed through the orientating nozzle and the effect on the fluorescence profile examined. Although bull spermatozoa have flattened heads, they did not display a biphasic scatter (size) profile similar to that seen when analysing chicken RBC. Nevertheless, the heads of bull spermatozoa could be positively orientated, since the resulting fluorescence profiles were monophasic and did not overlap; (Figure 5b). In contrast, the bimodal fluorescence distribution of intact bull spermatozoa stained with Hoechst 33342 was not altered by rotation of the nozzle (Figure 5c). The percentage of cells within each peak is shown in Figure 6.

Bull spermatozoa stained with Hoechst 33342 in milk or egg medium show a complex profile of fluorescence when analysed on the FACS. The observed fluorescence distribution of particles the size of spermatozoa (~2 x 5 x 10 µm head, 40 µm tail) can be divided into three main areas: (1) unstained material, (2) a pair of highly fluorescent peaks (B) shown to consist of dead or moribund spermatozoa, and (3) a pair of peaks (AI and AII) with intermediate fluorescence which consist of spermatozoa with normal forward motility. Attention has been concentrated on peaks AI and AII.

An increased staining of non-viable cells by Hoechst 33342 similar to that seen here for bovine spermatozoa has previously been reported for dead or dying lymphocytes stained with the same dye. It has been suggested (n) that the increased uptake of stain was due

to a breakdown of the integrity of the cell membrane at cell death. This may be the mechanism responsible for the observed increase in fluorescence of dead spermatozoa although it is possible that the normally tightly packed DNA in the nucleus becomes disorgaznized and this contributes to the increased staining. However, preliminary fluorometric studies suggest that a considerable increase in the fluorescence intensity of Hoechst 33342 occurs as the pH decreases, irrespective of whether the dye is bound to DNA, protein or is free in solution. This observation suggests that the B peaks may arise because of increased nuclear acidity at death.

The bimodal distribution observed in the Hoechst 33342 staining of viable spermatozoa (peaks A) is probably a consequence of the biologically different kinds of spermatozoa in the normal ejaculate. Accordingly a comparison of the fluorescence profiles of mammalian and bird spermatozoa, which are heterogametic and homogametic respectively shows the cockerel spermatozoa to have a unimodal distribution; Figure 5 illustrates that although the heads of spermatozoa can be orientated, the bimodal fluorescence distribution of Hoechst 33342-stained intact live spermatozoa is apparently independent of the orientation of the sperm heads around their long axis; and peaks AI and AII (Figure 4), although not clearly unimodal, are of predictable fluorescence in that spermatozoa separated from peak AII fluoresce more brightly than those from AI: a difference which averages at about 15%. If bimodality had a machine orientation artefact the separated population would be expected to have identical (bimodal) distributions.

Thus the observed bimodality of fluorescence distribution indicates the presence of two physiologically or biologically different sub-populations of viable spermatozoa. The sub-populations (AI and AII) may reflect spermatozoa at distinct stages of late

maturation or the difference between X- and Y- chromosome bearing spermatozoa. Experimental work with rabbits has yielded a 3.5:1 ratio of correct sex to incorrect sex, which is very close to the ratio which would be predicted from a theoretical estimate of the overlaps between the two sorted peaks. The above described method thus has a useful application in sorting spermatozoa according to whether they are X- or Y- chromosome bearing spermatozoa.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A method of sorting living spermatozoa, the method comprising: the vital staining of spermatozoa, with a fluorochrome dye; subjecting the stained spermatozoa to a light source which causes fluorescence; and sorting the spermatozoa according to the fluorescence intensities associated therewith.

2. A method according to claim 1, wherein the dye is a bisbenzimidazole dye.

3. A method according to claim 1 or 2, wherein the spermatozoa are of one of the following mammalian genera or families; bovidae; equidae; capridae; ovidae; lagomorphidae; and hominidae.

4. A method according to claim 1 or 2, when used to separate spermatozoa into different groups; one group mainly comprising X-chromosome bearing spermatozoa; and another group mainly comprising Y-chromosome bearing spermatozoa.

5. A method as claimed in claim 1, wherein the spermatozoa are sorted by a flow microfluormetric process.

6. A method of sorting living spermatozoa, the method comprising: the vital staining of spermatozoa with a fluorochrome dye which binds tightly to DNA, does not intercalate the DNA molecule of chromosomes and hence does not disrupt the structure of DNA molecules; subjecting the spermatozoa to a light source which causes fluorescence and sorting the spermatozoa into different groups according to the fluorescence intensities associated therewith, one group mainly comprising X-chromosome bearing spermatozoa; and another group mainly comprising Y-chromosome bearing spermatozoa.

7. A method according to claim 6, wherein the dye is a bisbenzimidazole dye.

8. A method according to claim 5 or 6, wherein the spermatozoa are one of the following mammalian genera or families; bovidae; equidae; capridae; ovidae; lagomorphidae; and hominidae.

9. A method as claimed in claim 6, wherein the spermatozoa are sorted by á flow microfluorometric process.

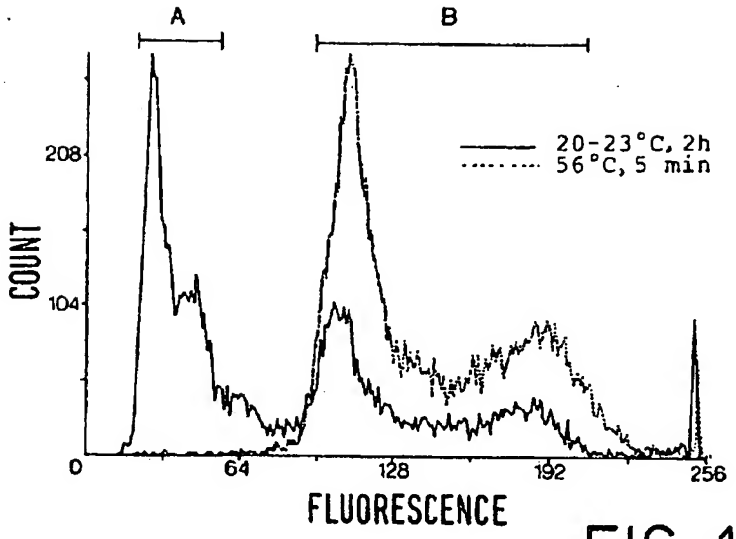
A
B
208
104
0
COUNT
64
128
192
256
FLUORESCENCE
20-23°C, 2h
56°C, 5 min

FIG. 1

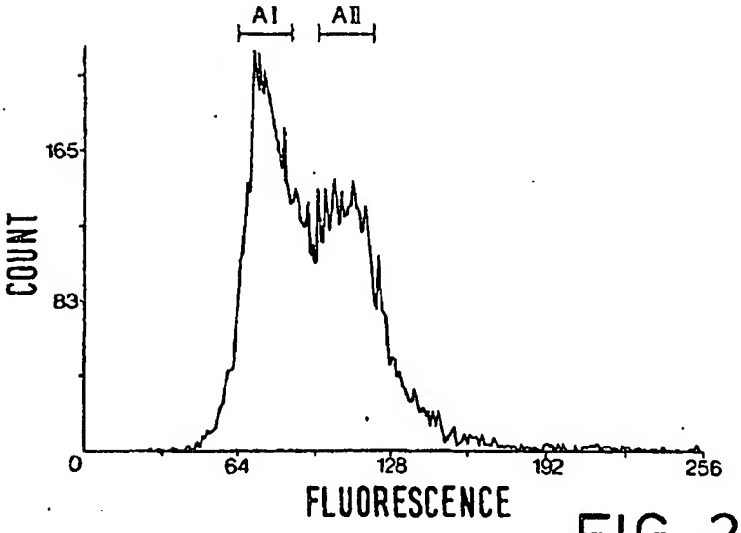
AI
AII
165
83
0
COUNT
64
128
192
256
FLUORESCENCE

FIG. 2

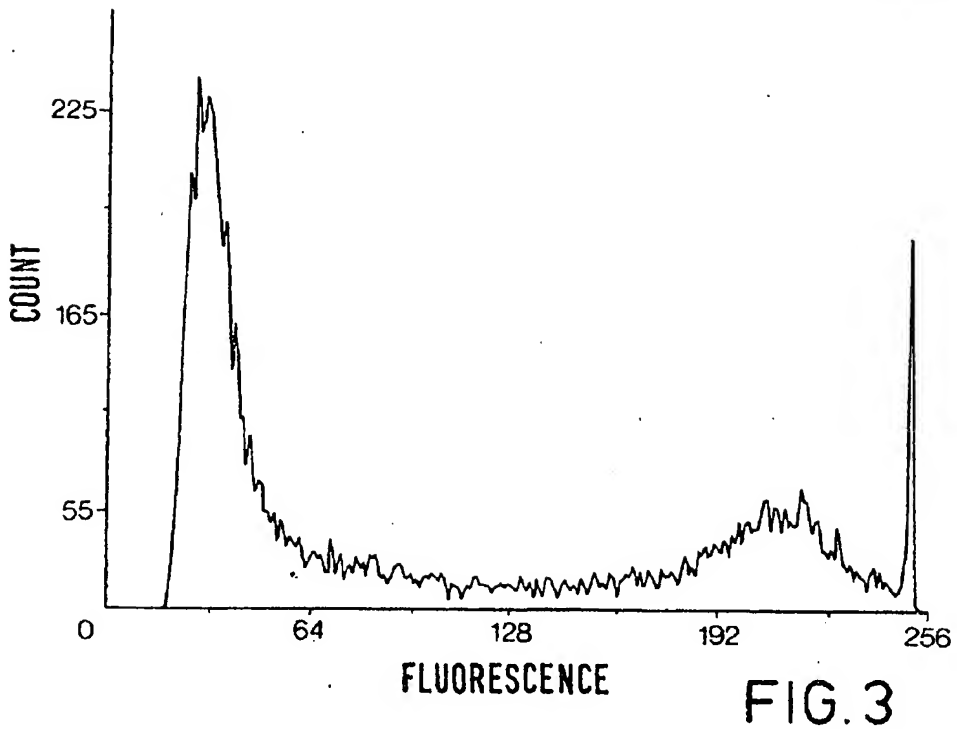
225
165
55
0
64
128
192
256
COUNT
FLUORESCENCE

FIG. 3

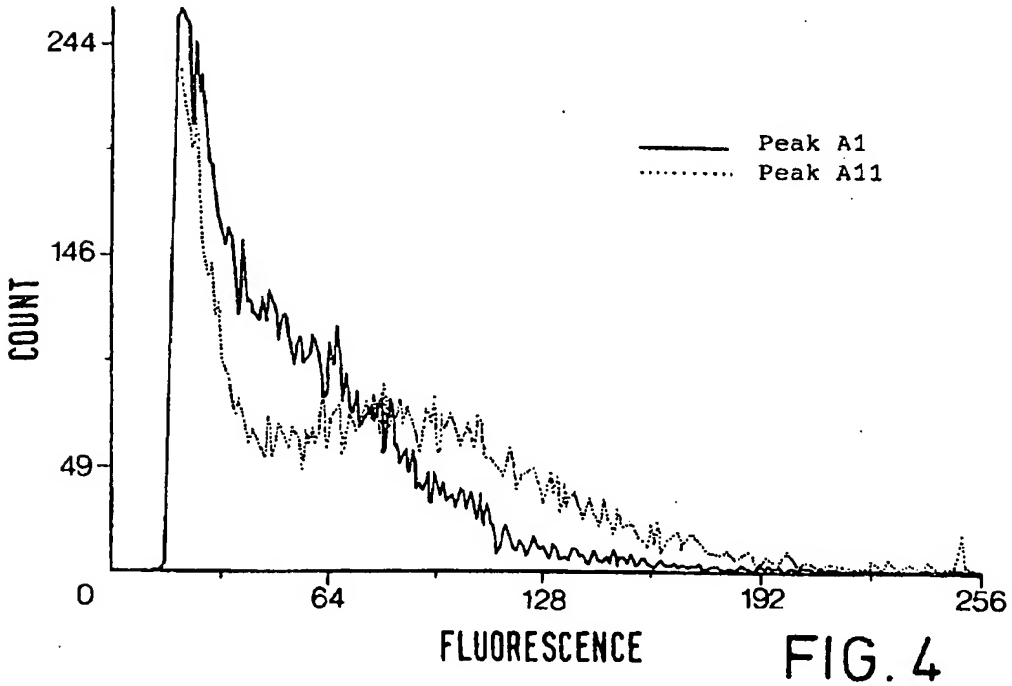
244
146
49
0
64
128
192
256
COUNT
FLUORESCENCE
Peak A1
Peak A11

FIG. 4

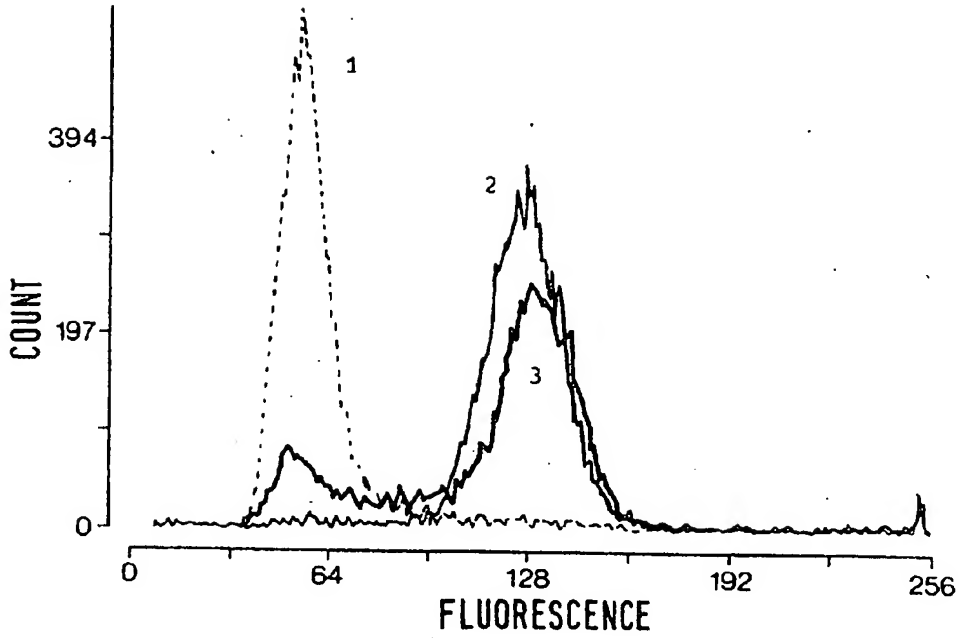

FIG. 5a

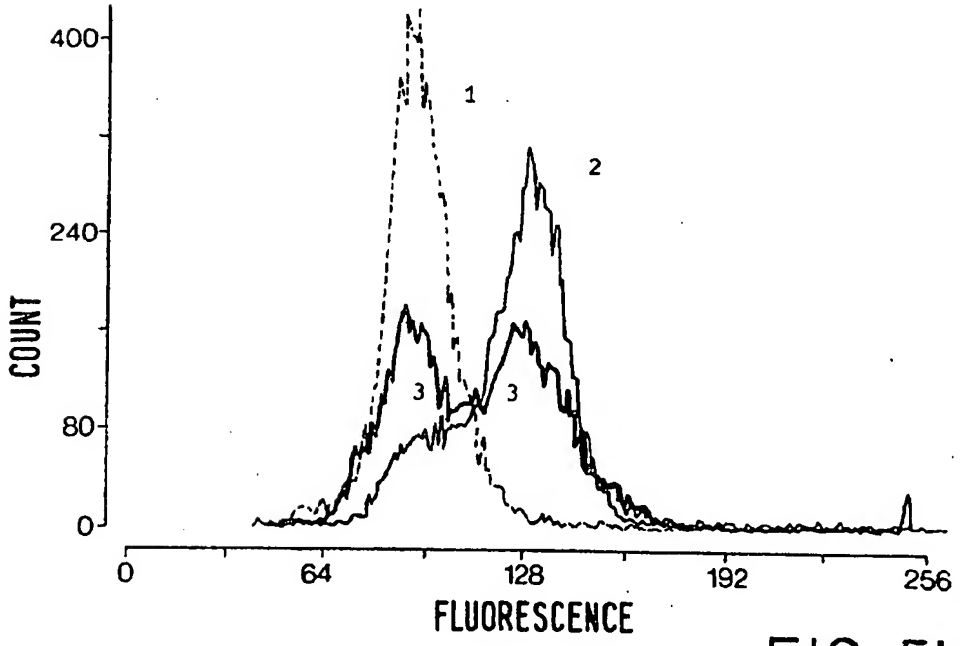

FIG. 5b

Siemens Aktiengesellschaft

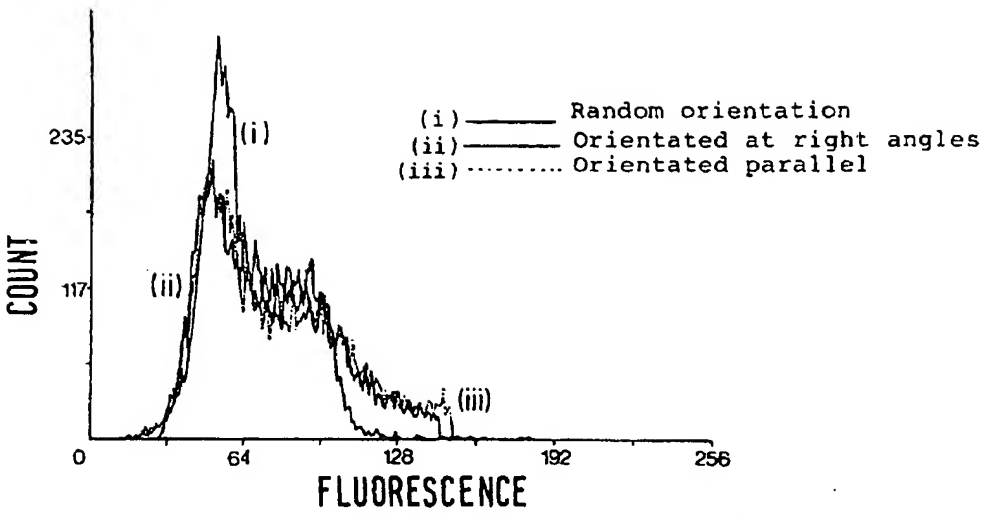

FIG. 5c

Orientation of cell to laser beam	Chicken RBC		Bull spermatozoa			
			Heads		Intact	
	Low Peak	High Peak	Low Peak	High Peak	Low Peak	High Peak
Random (normal nozzle)	22	78	43	57	51	49
Narrow side	94	6	90	10	59	41
Broad side	3	97	22	78	51	49

The values are the no. of cells in each peak of the distribution expressed as a % of the total.

FIG. 6